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Ejaculates are not used as nuptial gifts in simultaneously hermaphroditic snails

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Revised version sent to Zoology

Abstract

Promoted by sexual selection, males usually adopt different ways to increase their fertilization chances. In many insect taxa males donate nuptial gifts together with sperm, which represent a valuable additional nutrient source that females can use to provision eggs. This has also been suggested to occur for simultaneous hermaphrodites, organisms with both sex functions. In theory, donation of nuptial gifts or extra nutrients is not effective when these organisms mate reciprocally (mutual exchange of ejaculates), since each partner receives the amount it also transfers. Hence, the net amount gained would be zero, and considering the non-trivial costs of metabolic conversion the energy balance of this exchange ends up negative. To test this prediction, we measured material (dry weight) and resource (carbon and nitrogen content) investment into ejaculates of the fresh water snail *Lymnaea stagnalis* and spermatophores of the land snail *Cornu aspersum*. When compared to eggs, our results indicate that the investment is low for ejaculates and spermatophores, neither of which represent a significant contribution to egg production. Importantly, when these snails mated reciprocally, couples exchanged similar amounts of material and resources, thus a gain of extra substances seems irrelevant. Hence, caution is needed when generalizing functions of male reproductive strategies across mating systems. Although digestion of ejaculates does not provide extra material and resources in simultaneous hermaphrodites, their absorption could still be important to eliminate excess of received sperm and select sperm via cryptic female choice.

Introduction

When males are in competition for egg fertilization, different strategies that increase their reproductive success compared to rivals are advantageous and favoured by sexual selection (Kodric-Brown and Brown, 1984; Birkhead and Pizzari, 2002; Snook, 2005). One well known example is the donation of extra nutrients as a nuptial gift to a female, which is common in many taxa of insects (Thornhill, 1976; Vahed, 1998; Gwynne, 2008). The donated nutrients can be compounds transferred with sperm, such as seminal fluid, spermatophores, or lipids (Vahed, 1998; Lewis and South, 2012; Lewis et al., 2014), which are digested by use of enzymes released in the female genital tract (e.g., Hartmann and Loher, 1999) or in specialized organs (e.g., Reijden et al., 1997). Nutrients can also take the form of male body parts, such as a male's hind wing, caught prey or salivary secretions that are acquired by the female via oral ingestion before or during copulation (Lewis and South, 2012). Nuptial gifts can benefit males at different stages, before, during and after mating (reviewed by Lewis and South, 2012). After copulation male reproductive success can be enhanced by increasing female fecundity; for example, females of the moth species *Utetheisa ornatrix* produce 32 more eggs in their clutches after digestion of one spermatophore, which is 15% more than their usual egg output (LaMunyon and Eisner, 1994).

It has also been suggested that donated ejaculates could represent a source of additional nutrients to the partner or that they could be donated as a nuptial gift usable for egg production in simultaneous hermaphrodites (Charnov, 1979; Yusa, 1996; Greeff and Michiels, 1999b; Rogers and Chase, 2001; Evanno et al., 2005; Garefalaki et al., 2010; Sauer and Hausdorf, 2010; Lange et al., 2012; Yamaguchi et al., 2012; Kimura and Chiba, 2013b), i.e. organisms that have and use both male and female reproductive organs. However, whether such cases occur has not been tested yet, these are either speculations or simplifying assumptions (see also Schärer et al., 2014).

When sperm transfer is reciprocal in simultaneous hermaphrodites, i.e. both partners give and receive an ejaculate during one mating encounter, donation of nutrients seems unlikely to be effective (Koene and Chase, 1998b; Michiels, 1998).

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First, if the donated material is a nuptial gift, this could not only be used for egg production but also be invested in body maintenance and/or in the male function of the partner (Figure 5.1), which would be a disadvantage to the donor and could potentially benefit the recipient that is a rival in sperm competition (Michiels, 1998). Second, irrespective of this, when sperm transfer is reciprocal an individual will receive roughly the same amount of material and resources as it donates (Figure 5.1), even when sperm transfer is unilateral, an individual will donate and receive an equal number of times on average. Thus, the net substance gain would be zero for both partners (Beese et al., 2006a).

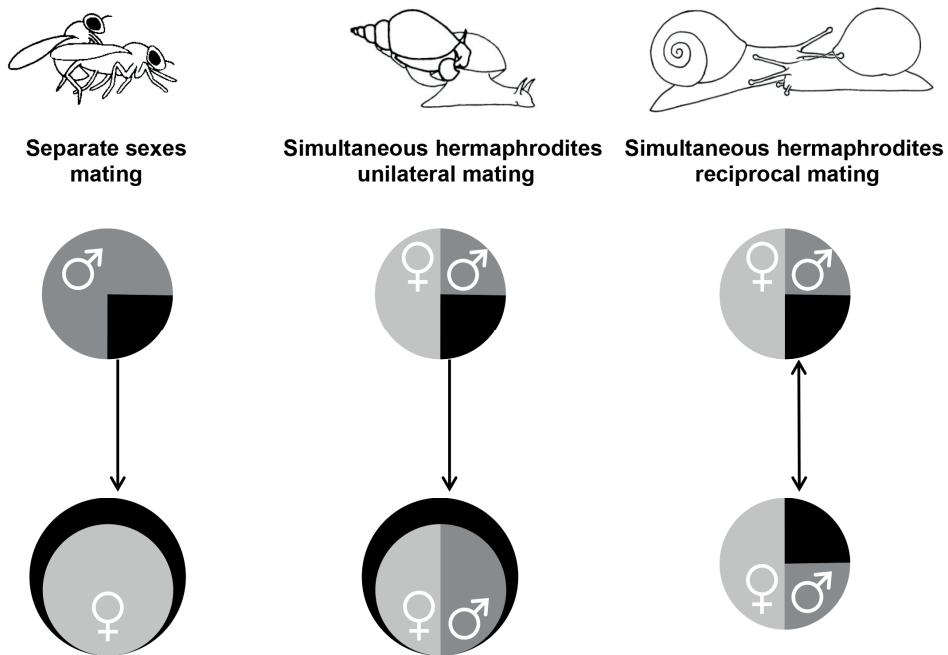


Figure 5.1 Fate of donated ejaculate (in black) between mating systems. In separate-sexed mating (e.g., *Drosophila*), the donated ejaculate is received and used only by the female. In simultaneous hermaphrodites, when mating is unilateral (e.g., *Lymnaea stagnalis*), the received ejaculate can contribute to the recipient's reproductive investment (male and/or female); when mating is reciprocal (e.g., *Cornu aspersum*), the amounts of male investment are being exchanged, hence the bidirectional arrow (so no net gain on either side). *Drosophila* drawing from Zizzari et al. (2014). Note that the conversion costs of the received ejaculate are not taken into account in this visualization. For example, in reality the exchange of the black portions in the right hand side of the figure would result in smaller usable black portions (i.e. what is left after the energy needed for metabolic conversion is subtracted).

More importantly, the costs of metabolic conversion of the received ejaculate into energy would result in an energy loss for the partner (Leonard, 1999). Despite these insights, the use of ejaculate digestion as a way of gaining a material benefit from a mating is a recurring suggestion in the literature on hermaphrodites. However, until now quantitative data are lacking on the amount of material and resources exchanged during mating to confirm this scenario. Thus, here, for the first time, we addressed this issue directly.

To test the abovementioned predictions, we used the fresh water snail *Lymnaea stagnalis* and the land snail *Cornu aspersum*, because they digest received ejaculates and represent two different cases of mating reciprocity that occur in simultaneously hermaphroditic organisms. Reciprocation for *L. stagnalis* is not obligatory, meaning that after a mating encounter, where one snail acted as female while the other performed the male role (hereafter named primary donor), either the snails separate or they swap roles: the one that was performing the female role can now act as male (hereafter named secondary donor) and inseminate the other. Thus, these snails can perform one sexual role at the time in a so-called sequential mating (Koene and Ter Maat, 2005). In contrast, mating reciprocation in *C. aspersum* is obligatory (Adamo and Chase, 1988; Chase and Vaga, 2006). During a mating encounter, both snails act as female and male at the same time by each receiving and donating a spermatophore. Hence, potential nutrient transfer is always mutual in *C. aspersum*, while it is unidirectional in *L. stagnalis* when reciprocation does not occur.

To investigate whether donated ejaculates and spermatophores could provide valuable extra substances for a mating partner, for each species we quantified male contribution by measuring material investment as dry weight and resource investment as carbon and nitrogen content. These values were compared to the material and resource investment in eggs to verify whether ejaculates and spermatophores could represent significant substances to be used for egg production. To determine if spermatophores and ejaculates could function as nuptial gifts, we assessed whether *C. aspersum* couples and primary and secondary sperm donors of *L. stagnalis* invested similarly during mating.

Material and methods

Lymnaea stagnalis

Adult snails, aged four months, were taken from the lab culture of the VU University Amsterdam, The Netherlands. They were fed *ad libitum* with lettuce and all isolated in individual perforated polythene jars (9 x 9 x 10 cm) placed in a tank with running low-copper water at 20°C and with a photoperiod of L:12h D:12h.

To estimate body size, shell length of egg-layers, primary and secondary sperm donors was measured with a digital calliper. To measure material investment into eggs and ejaculates compared to body mass, we measured egg and ejaculate dry weights after freeze-drying them for one day (ejaculates were freeze-dried during centrifugation). The dry body mass was calculated as the sum of the dry weight of the body without shell (immediately after mating or laying eggs, snails were anesthetized with 50mM MgCl₂, their bodies kept at -20°C and then freeze dried for two days) and the shell, which was removed after anesthesia and dried at 50°C in a stove for 1.5 h. To estimate resource investment, the percentage of carbon and nitrogen contained in egg masses and ejaculates were measured with a CN-analyser (FlashEA 1112 series). Each ejaculate could be analysed individually, whereas, due to the large size of egg masses, three portions per crushed egg mass were analysed and the average of those measurements considered. All samples were weighed before analysis, which allowed us to calculate the total amount of nitrogen and carbon content. Egg masses and ejaculates were collected as follows.

Egg mass collection

Lymnaea stagnalis from our lab culture lays 1–3 eggs masses per week (Nakadera et al., 2014a). Eggs are embedded in a gelatinous string and enclosed by a capsule to form an egg mass (Dogterom et al., 1983). To obtain eggs, snails were kept in isolation for four days in perforated polythene jars (9 x 9 x 10 cm) to allow for adjustment to experimental conditions and check for egg laying ability (Hoffer et al., 2010). After this period, they were transferred to a clean jar (9 x 9 x 10 cm) to stimulate egg laying (Ter Maat et al., 1983). Snails were checked daily for the

following three days during which the first egg mass laid by every individual was collected. The egg masses were scanned (CanoScan Lide 700F) so that the number of eggs could be counted with ImageJ. Afterwards, all egg masses were kept at -20°C.

Ejaculate collection

In order to collect the ejaculate of primary donors, snails were kept in isolation for one week to increase their willingness to mate in the male role (De Boer et al., 1997). After this week, each day snails were randomly paired up for the mating trials in polythene containers without perforations (9 x 9 x 10 cm). One snail of the pair was marked with nail polish on its shell to enable distinction between the two partners during the behavioural observations. Copulation behaviour was observed every ten minutes (for a visualization of the behaviours see Koene, 2010) and we noted which snail wanted to perform the male role when mounting the shell of the other; whether the male-acting snail was probing by everting its preputium; and whether snails were mating when the penis was inserted in the female gonopore. This observation was repeated until snails stopped mating. In order to collect the ejaculate, immediately after mating the sperm receiver was anesthetized with 50mM MgCl₂ because the donated ejaculate has not yet been moved to the bursa copulatrix for digestion (Loose and Koene, 2008). Thus, the vaginal duct was dissected out, placed in an Eppendorf with distilled water and vortexed for 1 min. to recover the ejaculate. If some of the ejaculate was still attached to the tissue we removed it with tweezers. The Eppendorfs containing the ejaculates were vortexed for 1 min. and centrifuged at 20,000 rpm for 15 min. to form a pellet at the bottom. Then, excessive water was removed until 0.1 ml was left. Samples were kept at -20°C.

For ejaculate collection of secondary donors, nearly the same protocol as above was followed. All snails were isolated for a week to stimulate mating reciprocation (Koene and Ter Maat, 2005). Instead of dissecting out the ejaculate of the first receiver when the mating stopped, we waited for the reciprocal mating to start. When it did, observation of the mating behaviour was conducted as previously

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described. After the second mating ended, ejaculate collection was conducted as explained above.

Cornu aspersum

Adult snails of the subspecies *Cornu aspersum maximum* were obtained from the snail farm Slow Escargot, Nieuwaal, The Netherlands. They were kept at 20°C and L:16h D:8h photoperiod at 60% humidity. They were cleaned, fed lettuce *ab libitum* and snail feed to provide calcium twice a week (the Chase mix, see Lodi and Koene, 2016a).

To estimate body size, shell volume of egg-layers and mating couples was determined by measuring length, height and width of the shell with a digital calliper and then calculated with the formula of Rogers and Chase (2001). To determine material investment compared to body mass, the dry weight of eggs and spermatophores was measured after they were freeze-dried for one day. Body dry mass was calculated as the sum of the dry weight of the body without shell (immediately after mating or laying eggs, snails were anesthetized with 50mM MgCl₂, their bodies kept at -20°C and then freeze dried for two days) plus the shell, which was removed after anesthesia and dried at 50°C in a stove for 1.5 h. To estimate resource investment, the percentage of carbon and nitrogen content of eggs and spermatophores was measured with a CN-analyser (FlashEA 1112 series). We randomly took three eggs per egg clutch (or fewer in case snails laid fewer eggs), crushed them individually and analysed a portion of each egg. For spermatophores, we divided each one in its three main components (neck, body, tail), crushed them individually and analysed a part of each. The average of the three egg parts and the three spermatophore parts, respectively, was considered. All samples were weighed before analysis, which allowed us to calculate the total amount of nitrogen and carbon content. Eggs and spermatophores were collected as follows.

Egg collection

Snails of *C. aspersum maximum* used in this study normally lay eggs twice a year (farm Slow Escargot, personal communication). Upon arrival, thirty snails were

grouped per cage (32 x 20 x 20 cm) for one week to give them the opportunity to mate to obtain sperm for egg fertilization and/or enough time for sperm received from previous matings to reach the sperm-storage organ (Rogers and Chase, 2001). Subsequently, snails were individually kept in plastic boxes (17.5 x 11 x 13 cm) with 4–5 cm of moist soil at the bottom to allow them to dig a hole in which to lay eggs (Koene and Chase, 1998b; Chase and Blanchard, 2006). Boxes were checked for eggs daily. When eggs were found, they were collected, counted and then kept at -20°C.

Spermatophore collection

Upon arrival, 116 snails were isolated for at least 10 days in plastic boxes (11.5 x 11.5 x 5 cm) with moist paper towel at the bottom, to increase their eagerness for courting (Adamo and Chase, 1990a). For every mating trial, snails were kept in groups of 14 individuals per glass cage (30 x 20 x 10 cm). When a couple started courting, i.e. extensive tentacle contact and biting (Adamo and Chase, 1988), it was transferred to a plastic box (17.5 x 11 x 13 cm) to allow for closer observation. This transfer did not affect the couples behaviour except in one case where courtship stopped. When mutual penis intromission occurs, the spermatophore is immediately formed (it does not contain sperm until 2-6 h later), and its transfer occurs only after 4.5–6 h (Adamo and Chase, 1988). Thus, approximately 5 hours after intromission, snails were separated by slowly pulling them apart and within an hour they ejected their own sperm-filled spermatophore via their genital pore (Lind, 1973; Koene and Chase, 1998b; Rogers and Chase, 2001; Chase and Vaga, 2006). Spermatophores were collected and kept at -20°C. In eight cases the tail of the spermatophore broke and the rest was removed by dissecting the snail under a stereo microscope.

Results

Lymnaea stagnalis

Mean \pm SD shell length was 2.94 ± 0.11 cm for egg-layers ($N=27$), 2.99 ± 0.09 cm for primary donors ($N=24$) and 3.05 ± 0.10 cm for secondary donors ($N=25$). Snails

of these three groups differed in shell length with egg-layers being shorter than the second donor group (ANOVA: $F_{2,73}=7.889$, $p=0.001$, Tukey HSD $p<0.001$). However, this difference is negligible since such size ranges are normally seen in snails of this age and their body masses did not differ (ANOVA: $F_{2,73}=2.607$, $p=0.081$). Moreover, in the light of adult shell lengths ranging from 1.8 to around 4.0 cm in this species, the above difference of about 1 mm is relatively small.

As material investment, the mean \pm SD dry weight was 0.40 ± 0.17 mg for the ejaculate of primary donors ($N=24$) and 0.43 ± 0.10 mg for the ejaculate of secondary donors ($N=25$), which did not differ significantly (Mann-Whitney U test: $U=356.000$, d.f.=1, $p=0.263$; Table 5.1). This investment did not correlate with shell length for either primary ($r=0.089$, $p=0.679$) or secondary donors ($r=0.094$, $p=0.656$). In contrast, egg masses weighed on average 13.76 ± 3.42 mg and contained 113 ± 19 eggs. Snails invested significantly less material into ejaculates compared to egg masses (Kruskal-Wallis test: $H=52.074$, d.f.=2, $p<0.001$; Bonferroni post-hoc test: $p<0.001$ when dry weight of eggs was compared to those of ejaculates of primary and secondary donors). Material investment of an egg mass compared to body mass was 3.41%, and only 0.10% for the ejaculates of both primary and secondary donors (Table 5.1).

In terms of resource investment, the amount of carbon did not differ between the ejaculates of primary ($N=24$) and secondary donors ($N=25$) (Mann-Whitney U test: $U=372.000$, d.f.=1, $p=0.150$), nor did the amount of nitrogen (Mann-Whitney U test: $U=374.000$, d.f.=1, $p=0.139$). Overall, the egg samples ($N=27$) had the largest quantity of carbon and nitrogen compared to the ejaculate samples (carbon: Kruskal-Wallis test $H=52.414$, d.f.=2, $p<0.001$; nitrogen: Kruskal-Wallis test $H=52.462$, d.f.=2, $p<0.001$; Bonferroni post-hoc test $p<0.001$ when both carbon and nitrogen of eggs was compared to those of ejaculates of primary and secondary donors) (Table 5.1). Percentages of carbon and nitrogen content of ejaculates of primary donors, secondary donors and egg masses are visible in Table 5.1. The number of ejaculates needed to provision enough carbon for one egg mass would be 33 and 30, respectively for primary and secondary donors, and 21 and 20, respectively, to provision enough nitrogen.

Cornu aspersum

Mean \pm SD shell volume was $11.96 \pm 1.90 \text{ cm}^3$ for egg-layers ($N=35$) and $12.60 \pm 1.71 \text{ cm}^3$ for mating couples ($N=13$), which did not differ (T-test: $F_{1, 59}=0.102$, $p=0.750$).

On average, the dry weight of one spermatophore ($N=26$) was $8.78 \pm 1.72 \text{ mg}$ and such an investment did not correlate with shell volume ($r=0.259$, $p=0.201$), nor with the size of the spermatophore received by the partner ($r=0.111$, $p=0.719$; snails of each couple were assigned randomly as partner number one or two). In contrast, the dry weight of an egg clutch ($N=35$) was $383.91 \pm 300.16 \text{ mg}$, which contained a mean of 50 ± 36 eggs. The dry weight of spermatophores was significantly lower compared to eggs (Mann-Whitney U test: $U=52.000$, $d.f.=1$, $p<0.001$). Material investment compared to body mass was 6.31% for egg clutches and only 0.13% for spermatophores (Table 5.1).

Concerning resource investment, the egg samples ($N=34$) had larger amounts of carbon (Mann-Whitney U test: $U=27.000$, $d.f.=1$, $p<0.001$) and nitrogen (Mann-Whitney U test: $U=92.000$, $d.f.=1$, $p<0.001$) compared to spermatophores ($N=26$) (Table 5.1). Percentages of carbon and nitrogen content of spermatophores and egg clutches are visible in Table 5.1. The number of spermatophores needed to provision enough carbon for one egg clutch would be 31, and to provision enough nitrogen it would be 14.

Within a mating couple, the lighter spermatophores transferred between two mating partners on average weighed $7.81 \pm 1.38 \text{ mg}$ and the heavier $9.76 \pm 1.49 \text{ mg}$, resulting in a difference in material investment of $1.95 \pm 1.28 \text{ mg}$. This difference represents 22.24% of the average investment into a spermatophore and 0.51% in an egg clutch. The average difference in resource investment between heavier and lighter spermatophores was $0.95 \pm 0.61 \text{ mg}$ for carbon and $0.28 \pm 0.19 \text{ mg}$ for nitrogen. This, respectively, represents 22.35% and 22.69% of the average amount contained in one spermatophore and 0.72% carbon and 1.57% nitrogen in an egg clutch.

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Table 5.1 Mean \pm SD investment into egg masses and ejaculates of *L. stagnalis* and into egg clutches and spermatophores of *C. aspersum*.

	<i>Lymnaea stagnalis</i>			<i>Cornu aspersum</i>	
	Egg mass	Primary donor ejaculate	Secondary donor ejaculate	Egg clutch	Spermatophore
Dry weight (mg)	13.76 \pm 3.42	0.40 \pm 0.17	0.43 \pm 0.10	383.91 \pm 300.16	8.78 \pm 1.72
% Sample to body mass	3.41	0.10	0.10	6.31	0.13
C %	44.33 \pm 1.08	45.35 \pm 3.58	46.68 \pm 4.70	33.72 \pm 2.32	48.41 \pm 0.42
N %	6.39 \pm 0.26	10.29 \pm 1.00	10.49 \pm 1.38	4.60 \pm 0.70	13.98 \pm 0.31
C (mg)	6.11 \pm 1.57	0.18 \pm 0.08	0.20 \pm 0.05	132.14 \pm 99.32	4.25 \pm 0.84
N (mg)	0.88 \pm 0.23	0.04 \pm 0.02	0.05 \pm 0.01	17.80 \pm 13.50	1.23 \pm 0.25

Discussion

There is a lack of empirical evidence for whether simultaneous hermaphrodites gain a nutritional benefit from the receipt of ejaculates during mating. So far, only one study quantified male material and resource investment in a land snail, but since the research aim was focused on something else the net substance gain when mating is reciprocal was not measured (Locher and Baur, 2000). Our results show that ejaculates of the fresh water snail *L. stagnalis* and spermatophores of the land snail *C. aspersum* do not represent a substantial contribution to the partner. When compared to the investment in eggs, the male investment is considered too low to contribute meaningfully to the provisioning of eggs. In addition, when mating is reciprocal, couples exchange similar amounts of material and resources, making donation of a nuptial gift unlikely to evolve since the net substance gain for a partner is almost zero.

In general, the relative material investment into ejaculates and spermatophores of the species used in this study is very low, only 0.10% compared to body mass for both primary and secondary donors of *L. stagnalis* and 0.13% for *C. aspersum*. Compared to other species, this is similar to the low investment of the male ground beetle *Carabus insulicola*, which transfers a spermatophore of 0.61% his body mass that is considered less of a nutrient source for females compared to other insect species (Takami, 2002). Higher contributions are found in the seed beetle *Stator limbatus* that gives an ejaculate that equals 4.8% of male body mass (Fox et

al., 1995), and in the Lepidoptera *Pieris rapae*, where the male gives a spermatophore that can reach up to 7% of its body mass (Watanabe and Sato, 1993). Supplying even more, male moths of the species *Gluphisia septentrionis* invest 9% of their body weight in a spermatophore (Smedley and Eisner, 1996), male monarch butterflies invest 10% (Oberhauser, 1989), and tettigoniid males *Requena verticalis* invest up to 20% (Davies and Dadour, 1989). In all these species males could actually provide a valuable nutritional contribution, which is considered a nuptial gift. The mentioned examples concern male investment of one mating encounter, and often involve a specialized nuptial gift, but even if males mate multiple times and only donate ejaculates their contribution can remain high. For example, male seed beetle (*Callosobruchus maculatus*) that mate three times give an ejaculate that is on average 3% of his body mass each time (Savalli and Fox, 1999). In general, the large investment of males can be costly, for example, males of the genus *Ephippiger* lose 25% of their weight after spermatophore production (Thornhill, 1976) and males of the tropical bruchid, *Caryedon serratus* lose 15% (Boucher and Huignard, 1987). In contrast, based on our results, the low male material investment of *C. aspersum* and *L. stagnalis* seems much less costly. However, one has to keep in mind that male contribution to reproduction is not only defined by the quantity of material invested. Significant reproductive costs are also made for courtship, mating behaviour (e.g., dumpling squid *Euprymna tasmanica*; Franklin et al., 2012) and accessory gland product production (e.g., seminal fluid; Koene, 2017).

The investment of the male function of the snail species investigated here in terms of resources (measured as carbon and nitrogen content) was also relatively low, especially when compared to the amount necessary for producing eggs, excluding the metabolic conversion costs of the received material (Leonard, 1999). On average, 20 ejaculates would need to be digested by *L. stagnalis* in order to provide enough nitrogen to build an egg mass and 32 ejaculates to provide enough carbon. Similarly, for *C. aspersum* 14 spermatophores are needed to gain enough nitrogen and 31 for carbon to produce one egg clutch. The resource investment of *C. aspersum* reported here is similar to the concentration of nitrogen and carbon found in the eggs and spermatophores of the land snail *Arianta arbustorum*

(Locher and Baur, 2000). So, while for many insects species, partner-derived nutrients are known to be incorporated into eggs (e.g., Boggs and Watt, 1981) and largely affect female reproductive output in terms of egg size (e.g., Fox et al., 1995) and number of eggs laid (e.g., LaMunyon and Eisner, 1994), such a nuptial gift effect does not seem to apply to the snail species used in the current study.

The general low male investment reported here was similar between reciprocating mating partners. For *L. stagnalis*, a secondary donor donated a similar amount to what it received in terms of material and resources, i.e. primary donors did not invest more than secondary donors. Still, for *L. stagnalis* a gain of extra substances could be possible whenever reciprocation does not take place, which can happen when insufficient seminal fluid is available (Koene and Ter Maat, 2005). However, given the small amount received by the partner (0.40 mg) this would probably still not provide enough material and resources to really benefit from digesting this ejaculate.

According to Nakadera et al. (2014b), a difference in investment could have been expected between ejaculates of primary and secondary donors, with the second one being lower. Secondary donors are known to transfer significantly fewer sperm (61% reduction) in their next copulation due to the effect of seminal fluid proteins received in the ejaculate from the primary donor (Nakadera et al., 2014b). Hence, in our experiments this decline in sperm transfer might have been reflected in a lower investment compared to primary donors, but such a difference was not found. Probably, if the seminal fluid is the largest part of the ejaculate, proportionally less sperm may not be so noticeable.

Similarly, in the land snail *C. aspersum*, where mutual sperm exchange always occurs because mating reciprocation is obligatory (Adamo and Chase, 1988; Chase and Vaga, 2006), male investment of mating couples was similar. Within a pair, when a partner received a larger spermatophore compared to the one donated, it only gained approximately one fifth more material and resources. Hence, again a gain of extra substances via this route seems not to be meaningful. In these snails, accessory gland proteins are also transferred between partners in the form of mucous products on the surface of the so-called love-dart, which is a calcareous stylet that is stabbed through the partner's body wall during courtship

(reviewed by Lodi and Koene, 2016b). However, receipt of such mucous products does not affect the number of sperm delivered (Chase and Vaga, 2006), but instead reduces the number of sperm digested by the receiver (Rogers and Chase, 2001), which is why in our study we did not account for whether a snail was hit by a dart.

Important to note is that in our study we only quantified carbon and nitrogen content. This might not be sufficient to fully address the value of male resource investment. However, these measurements do cover the major components of male as well as female resource investment into reproduction. For *L. stagnalis* the ejaculate is made up of spermatozoa and seminal fluid proteins (Hoffer et al., 2010) and eggs primarily consist of proteins (60%), which together with galactogen represent 94% of the egg composition, whereas lipids, calcium and glycogen are almost absent (Wijsman and van Wijck-Batenburg, 1987). For *C. aspersum*, spermatophores are mainly made of spermatozoa surrounded by a protein case (Mann, 1984) and eggs, covered by a partially calcified shell, contain galactogen (38% in *Helix pomatia*) and calcium, while lipids are nearly absent (Tompa, 1984). For land snails, calcium is known to be an important factor in reproduction (Crowell, 1973). However, the amount of calcium in one spermatophore of *C. aspersum* (equal to 0.018 mg) is known to be irrelevant compared to the amount required for an egg clutch (equal to 24.25 mg) and thus unlikely to be donated as a nuptial gift (Koene and Chase, 1998b). Future research in other simultaneous hermaphrodites should aim to quantify other components transferred with sperm whenever relevant for the species studied.

To conclude, caution is needed when generalizing male reproductive strategies between mating systems. We suggest that nuptial gifts can be donated whenever the exchange is unilateral, e.g., from a male to a female (Figure 5.1). In contrast, in many simultaneous hermaphrodites sperm transfer is bilateral (Baur, 1998), making the net benefit for a partner effectively absent when material and resources exchange is similar (Figure 5.1). In addition, for these organisms the metabolic conversion of the received ejaculate into nutrients that can be invested in eggs is more costly than just producing more eggs themselves (Michiels, 1998; Leonard, 1999; Koene, 2017). Alternatively, producing a nuptial gift could require more

energy than the amount gained by ejaculate digestion (Koene et al., 2006). Even if the donated ejaculate seems not to provide a valuable source of extra material and resources for simultaneous hermaphrodites, its digestion could still be important to eliminate excess of received sperm since females only need a small fraction for fertilizing eggs while the rest needs to be discarded (Dewsbury, 1982; Ridley, 1988; Greeff and Michiels, 1999b). Moreover, ejaculate digestion allows for storage of sperm from different males in the sperm-storage organ since the space available is limited (Beese et al., 2006a), and it enables a partner to remain in control of fertilization processes if it eliminates sperm from low-quality mates (Eberhard, 1996). These functions may represent the main evolutionary driving force behind the evolution of sperm digestion in simultaneous hermaphrodites.

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